DEC 4 1923

THIS NUMBER COMPLETES VOLUME VIII

VOLUME VIII NUMBER 6

JOURNAL OF BACTERIOLOGY

OFFICIAL ORGAN OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NOVEMBER, 1923

EDITOR-IN-CHIEF
C.-E. A. Winslow

It is characteristic of Science and Progress that they continually open new fields to our visions.—PASTEUR.

PUBLISHED BI-MONTHLY
WILLIAMS & WILKINS COMPANY
BALTIMORE, U.S.A.

Entered as second-class matter April 6, 1916 at the postoffice at Baltimore, Maryland, under the act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in Section 1103, Act of October 3, 1917. Authorized on July 16, 1918

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PHYSIOLOGICAL STUDY OF AZOTOBACTER CHROOCOCCUM, BEIJERINCKII AND VINELANDII TYPES

UNOKICHI YAMAGATA AND ARAO ITANO

Department of Microbiology, Massachusetts Agricultural Experiment Station, Amherst, Massachusetts

Received for publication January 18, 1923

INTRODUCTION

This investigation deals with the relation of hydrogen ion concentration in specially prepared culture solution, to the growth of three types of Azotobacter, namely, Chroococcum, Beijerinckii, and Vinelandii.

A description of the organisms used, as well as some special technic developed by the authors, is noted in detail.

METHOD OF PROCEDURE

1. Determination of hydrogen ion concentration

The electrometric method which was described by Itano[1] and later modified slightly by the same author, was used throughout the investigation. The method was modified so that direct titration as well as direct reading of pH was made possible.

The modifications were as follows:

a. The Hildebrand electrode was used in place of Hasselbach's.

b. The Kohlrausch bridge was used for the meter bridge.

c. The sliding contact rheostat of 100 ohms resistance was injected in the battery circuit and adjusted at the start so that the current from the battery against the normal element balanced at 700 on the bridge wire. In other words, the apparatus was set so that 1.0189 volt against the battery read at 700 on the

[1] Itano, A. Bulletin 167, Mass. Agri. Exper. Station, Amherst, Mass.

bridge. On this basis the following circular disc with a pointer was constructed so that pH can be read directly from the bridge reading (graph I). This modification eliminates much calculation. It is, however, necessary to use the former method wherever such factors as gas tension, temperature and barometric corrections are to be considered.

Although the colorimetric method has been used very often by other investigators, the authors, realizing its inadequacy, especially in connection with the pigmenting organisms, used the electrometric method described above. It was also found that the "salt error" plays an important rôle in such a synthetic medium as Ashby solution.

II. Culture medium and adjustment of its hydrogen ion concentration

Ashby solution of the following composition was used:

Mannitol	20.0 grams
KH_2PO_4	0.2 gram
$MgSO_4 \cdot 7H_2O$	0.2 gram
NaCl	0.2 gram
$CaSO_4 \cdot 2H_2O$	0.1 gram
H_2O	1000 cc.

$CaCO_3$ was not added except in case of comparative study as control.

The pH was determined by titrating electrometrically with N/10 HCl and N/10 NaOH as in the results noted below:

To prepare the media of different pH, the following proceedure was adopted:

1. Ashby solution ($-CaCo_3$) of double strength was prepared.
2. To this, the determined amount of H_2O, N/10 HCl or N/10 NaOH and 10 cc. of inoculum was added so that the total volume became equal as noted in table 1.

III. Organisms used in the investigation

Three types of Azotobacter of the description given in table 2 were employed.

These organisms were isolated by the author's method[2] which was found to be very effective in obtaining pure Azotobacter free from *B. radiobactor*.

About 1 gram of soil was placed in 20 cc. Ashby solution, in a 150 cc. Erlenmeyer flask and incubated about three days. (The length of incubation varies for different types.) As soon as the medium became turbid, a loopful of the culture was

TABLE 1

Final preparation of the media of different pH

DESIRED pH	NUMBER OF					TOTAL VOLUME	FOUND pH
	Double strength medium	Inoculation	N/10 HCl	N/10 NaOH	Sterile distilled water		
	cc.	cc.	cc.	cc.	cc.	cc.	
Control*	90	10			100.0	200	7.0
4.5	90	10	0.2		99.8	200	4.0
5.0	90	10			100 0	200	5.0
5.5	90	10		0.5	99.5	200	6.0
6.0	90	10		1.0	99.0	200	6.3
6.4	90	10		1.3	98.7	200	6.6
6.6	90	10		1.6	98.4	200	6.7
6.8	90	10		1.7	98.3	200	6.8
7.0	90	10		2.0	98.0	200	7.0
7.5	90	10		2.4	97.6	200	7.4
8.0	90	10		3.0	97.0	200	7.6
8.5	90	10		3.5	96.5	200	8.0
9.0	90	10		4.2	95.8	200	8.3
10.0	90	10		5.8	94.2	200	9.2
11.0	90	10		12.0	88.0	200	10.3

* Contains $CaCO_3$ while all others, none.

plated out and the colonies were fished out and examined for Azotobacter in Meissner solution as usually employed. Further the growth of the organism in Ashby solution and the nitrogen fixation were tested. The organism thus obtained was acclimatized just before the inoculation.

The distribution of these organisms, in relation to the reaction of the soils examined, is noted in table 3.

[2] This method was perfected by Yamagata in collaboration with K. Aoi.

TABLE 2

Description of organisms

	NAME OF ORGANISMS		
	Chroococcum type	Beijerinckii type	Vinelandii type
Form and size	Short plump rod, gen. in pairs. 2.3x3.4	Large oval. 2.3x4.7	Oval. 1.8 x 3.4
Motility	Motile, in young culture only	No	Motile
Coloràtion on Ashby agar (28 to 30°C.)	A. White B. Brownish black	White Sulfur-yellow	White Greenish yellow: soluble
Coloration in Ashby solution (28 to 30° C.	A. White, turbid B. Brownish black surface film	White, turbid White sediment, pellicle formation	Greenish yellow Yellowish pink
Colony on Ashby agar plate (28 to 30°C.)	Round, pasty, dark concentric ring in the center	Somewhat round moist, wrinkled surface	Round, semi-transparent
Nitrogen fixation per gram mannit	6.96 mgm.	5.58 mgm.	9.12 mgm.

A, young culture; B, old culture.

TABLE 3

Distribution of Azotobacter

REACTION OF SOIL	NUMBER OF SOIL SAMPLES EXAMINED	NUMBER OF SOILS CONTAINED AZOTOBACTER		
		Azotobacter chroococcum type	*Azotobacter Beijerinckii* type	*Azotobacter vinelandii* type
Acid	119	0	0	0
Neutral	76	3	11	0
Slightly alkaline	62	26	20	0
Alkaline	43	37	0	3
Sum	300*	66	31	3

* These samples represent various cultivated soils in Japan, covering the country from the extreme north to the south.

The table indicates the distribution of Azotobacter relative to the reaction of the soil. *Azotobacter chroococcum* is most widely distributed and in the alkaline soils; *Azotobacter Beijerinckii*, is less common and in nearly neutral soils; and *Azotobacter vinelandii*, least common and in the alkaline soils.

Again, the geographical distribution of these organisms, especially of chroococcum, is worthy of note. They are found generally in warm climates where naturally the amount of humus is

TABLE 4

Growth of Azotobacter chroöcoccum type

NUMBER OF FLASK	ORIGINAL pH	RATE OF GROWTH			FINAL pH, 96 HOURS	NUMBER OF BACTERIA IN 20 CC. MEDIUM† 96 HOURS INOCULUM, 190,000 (NUMBER IN THOUSANDS)
		48 hours	96 hours	240 hours		
Control*	7.0	+	+	+	7.2	484,000
1	4.0	−	−	−	4.0	190
2	5.0	−	−	−	5.5	210
3	6.0	−	−	−	5.8	10,000
4	6.3	−	±	+	6.0	80,000
5	6.6	±	+	+	6.2	108,000
6	6.7	±	+	+	6.3	116,000
7	6.8	+	+	+	6.3	120,000
8	7.0	+	+	+	6.4	128,000
9	7.4	+	+	+	6.7	136,000
10	7.6	+	+	+	6.8	148,000
11	8.0	+	+	+	6.9	176,000
12	8.3	+	+	+	6.9	156,000
13	9.2	+	+	+	6.9	148,000
14	10.3	+	+	+	8.0	140,000

−, no growth; ± doubtful growth; +, positive growth.

* Contains $CaCO_3$ while all other flasks contained solution without it as has been stated previously.

† Breed Direct Counting method was used. Breed, R. S., Centbl. Bakt. Abt. II, 1911, vol. 30, 337–340.

almost lacking and the soil is neutral or alkaline, and very few in cold climates except where wood ash or $CaCO_3$ is applied, or where sea shell deposits are found. That means, in general, that the acid condition exists where the humus is present in abundance and is lacking in the alkaline salts, thus preventing the growth of Azotobacter, and it is especially true with *A. chroococcum* on account of its association with alkali.

INOCULATION OF MEDIA

One cubic centimeter of forty-eight hour old culture in Ashby solution ($-CaCO_3$—reaction adjusted to pH 7.0 with N/10 NaOH) was transferred into 19 cc. of the culture solution of respective pH so that the total volume became 20 cc. in every case.

TABLE 5

Growth of Azotobacter Beijerinckii type

NUMBER OF FLASK	ORIGINAL pH	RATE OF GROWTH			FINAL pH, 96 HOURS	NUMBER OF BACTERIA IN 20 CC. MEDIUM / 96 HOURS / INOCULUM, 122,000 (NUMBERS IN THOUSANDS)
		48 hours	96 hours	240 hours		
Control	7.0	+	+	+	7.1	164,000
1	4.0	−	−	−	4.1	122
2	5.0	−	−	−	5.2	200
3	6.0	−	−	−	5.8	5,600
4	6.3	−	+	+	5.9	15,600
5	6.6	−	+	+	6.3	20,800
6	6.7	−	+	+	6.5	45,000
7	6.8	−	+	+	6.5	62,200
8	7.0	+	+	+	6.5	69,000
9	7.4	−	+	+	6.8	50,000
10	7.6	−	+	+	6.8	45,200
11	8.0	−	+	+	7.0	18,200
12	8.3	−	−	+	7.5	8,000
13	9.2	−	−	+	7.8	3,000
14	10.3	−	−	±	8.4	400

RESULTS

The results are noted in table 4.

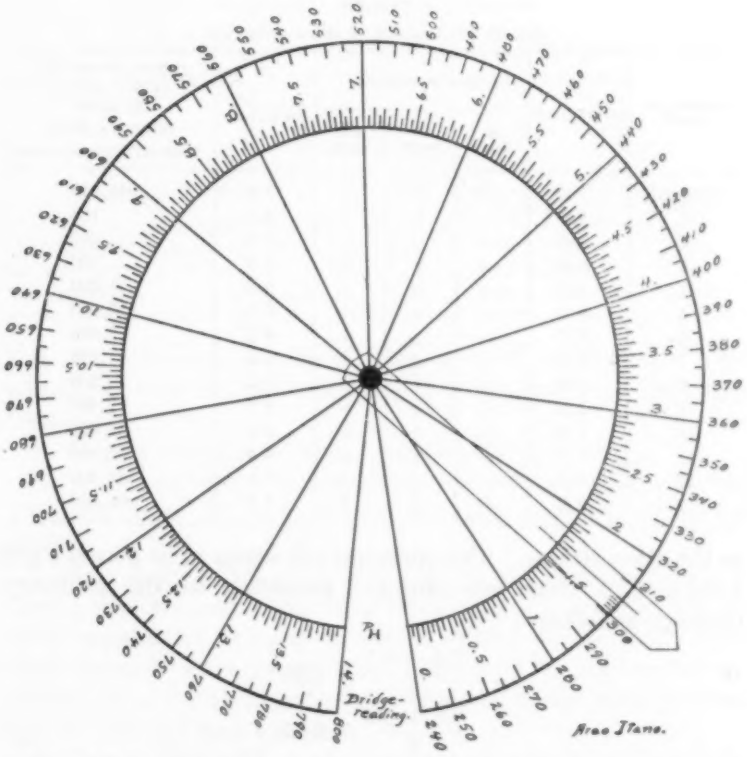
Table 4 indicates that the reaction of the media was changed more or less toward acid during the growth of the organism. The greater change took place on the alkaline side. It is also apparent that better growth took place in media of pH 7.0. A marked beneficial effect of $CaCO_3$ on growth is indicated by the fact that in the control, Ashby solution with $CaCO_3$, the organism grew to 484 millions while the maximum growth was 176 millions in the others. It is interesting to note that pH 7.0 has a slight inhibitory effect. The optimum pH seems to lie be-

tween 7.45 to 7.60, which was calculated by the authors adopting an arbitrary method as indicated below:

$$\frac{8.0 + 6.9}{2} = 7.45$$

$$\frac{8.3 + 6.9}{2} = 7.60$$

Graph 1.



where 6.9 represents pH where the inhibitory action was manifested somewhat markedly; 8.0, where the maximum growth took place; and 8.3, where the decrease in growth was indicated.

Table 5 indicates that the rate of growth is more markedly influenced at different pH values than is the case with the other organisms. For instance, at pH 7.0 good growth *of A. Beijerinckii* took place at the end of forty-eight hours while at all other reactions the organisms were inhibited. The inhibitory influence seems to increase with variations above or below pH 7.0. Only slight difference exists between the control and flask 8 where the pH is optimum. This seems to indicate that this organism is not influenced by the presence of $CaCO_3$ or at least not so much

TABLE 6

Growth of Azotobacter vinelandii type

NUMBER OF FLASK	ORIGINAL pH	RATE OF GROWTH			FINAL pH, 96 HOURS	NUMBER OF BACTERIA IN 20 CC. MEDIUM / 96 HOURS / INOCULUM, 160,000 (NUMBERS IN THOUSANDS)
		48 hours	96 hours	260 hours		
Control	7.0	+	+	+	7.0	944,000
1	4.0	−	−	−	4.0	160
2	5.0	−	−	−	5.3	300
3	6.0	−	−	+	5.9	500
4	6.3	−	±	+	6.0	1,200
5	6.6	−	±	+	6.3	10,800
6	6.7	−	±	+	6.3	17,600
7	6.8	−	±	+	6.5	21,400
8	7.0	±	+	+	6.5	22,200
9	7.4	+	+	+	6.8	28,000
10	7.6	+	+	+	6.9	36,000
11	8.0	+	+	+	7.0	72,000
12	8.3	+	+	+	7.1	64,000
13	9.2	+	+	+	7.3	60,000

as the other strains. The optimum pH seems to lie between pH 6.65 to 6.75 which was obtained according to the arbitrary methods as follows:

$$\frac{7.0 + 6.5}{2} = 6.75$$

$$\frac{6.8 + 6.5}{2} = 6.65$$

Table 6 indicates that the behavior of *A. vinelandii* is similar to that of *Azotobacter chroococcum* except the inhibitory action

by pH below 7 was slightly greater. Much greater beneficial effect of $CaCO_3$ is noted, viz., 944 millions in the control and 72 millions in flask no.11. The optimum pH seems to lie between pH 7.5-7.7 which was determined as follows:

$$\frac{8.0 + 7.0}{2} = 7.50$$

$$\frac{8.3 + 7.1}{2} = 7.70$$

SUMMARY AND CONCLUSIONS

1. The culture solution used in this investigation, Ashby solution without $CaCO_3$, served the purpose very well.

TABLE 7

AZOTOBACTER	pH	
	Optimum	Limiting
Chroöcoccum	7.45 to 7.60	5.80
Beijerinckii	6.65 to 6.75	5.80
Vinelandii	7.50 to 7.70	5.90

2. By preparing media of different pH as described, the amount of food and concentration of other ions were kept uniform.

3. Itano's modified method made the titration easy.

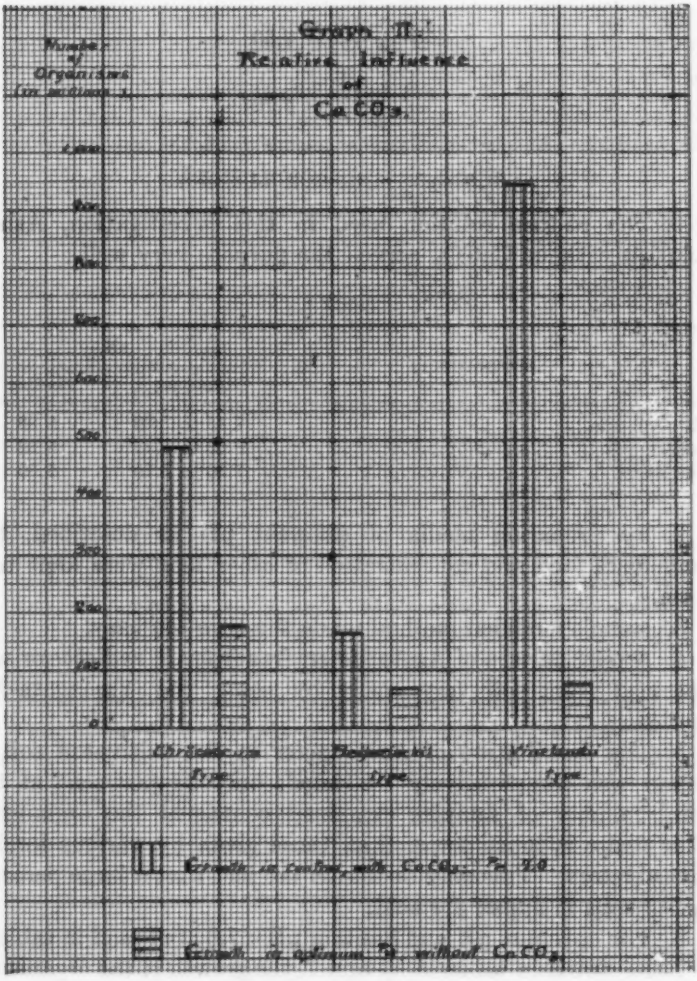
4. A pure culture of Azotobacter, free from *B. radiobactor* was obtained from soil very easily by using the Yamagata-Aoi method.

5. The distribution of Azotobacter in the cultivated soil in Japan was comparatively restricted; only 33 per cent of the total number of samples examined contained the organisms. This figure is much lower than those given by various foreign investigators. This may be due to the fact that a large percentage of soils in Japan are acid.

6. The optimum pH and limiting pH < 7.0 are found in table 7.

7. As noted in tables 5, 6, and 7, the growth of the organisms, at different pH, and after various periods seem to vary greatly.

That is, at the end of forty-eight hours, the growth of *A. chroococcum* took place at all pH's above 6.6; *A. Beijerinckii*, only in pH 7.0; *A. vinelandii*, at all pH's above 7.0.



8. The influence of calcium carbonate on Azotobacter seems to vary with the species. The Beijerinckii type grew very well in the absence of $CaCO_3$ while the other two types, especially vinelandii, are very sensitive. This is shown graphically in graph II.

9. At the end of ninety-six hours, the following different degrees of toleration of hydrogen ion concentration, by each organism, were observed: viz., *A. chroococcum* grew distinctly at all pH's above 6.6; *A. Beijerinckii*, from pH 6.3 to pH 8.0; *A. vinelandii*, at all pH's above 7.0. At the end of two hundred and forty hours, *A. chroococcum* and *A vinelandii* behaved similarly, or both organisms grew distinctly in all pH's above 6.0 while *A. Beijerinckii* grew from pH 6.3 to pH 9.2. Further investigation to determine the influence of OH ions on Azotobacter, in presence of $CaCO_3$ is in progress.

A preliminary investigation on some phase of this experiment was carried out by the colorimetric method under Prof. J. K. Wilson of Cornell University, to whom the authors are indebted.

ANTIGENIC RELATIONSHIPS OF BACT. TYPHOSUM

I. AGGLUTINATION[1]

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Received for publication February 1, 1923

The essential identity of antigenic structure of strains of *Bact. typhosum* is frequently brought into question by reports of "atypical" strains of typhoid bacilli and also by the reported success of attempts to divide this group of organisms into so-called "types" or "groups." Opposed to the latter conception is the generally accepted opinion that most, if not all, strains of *Bact. typhosum* are much alike and present marked cultural and antigenic uniformity.

The present work was prompted by the isolation from the urine, blood, or feces of several patients, some of whom were thought to have typhoid fever, of Gram-negative bacilli which, because of their source and cultural characteristics, apparently belonged to the typhoid-paratyphoid group. These strains failed to agglutinate with, or absorb agglutinin from, the laboratory sera. On being tested with sera prepared from two recently isolated typhoid strains some of the cultures were identified as true typhoid organisms. This observation suggested a study of the antigenic relationships of a large number of strains of *Bact. typhosum;* particularly as similar investigations had previously led to discordant conclusions and in some instances were based on scanty material.

The problem, presented, then, was to determine whether or not typhoid strains are alike or dissimilar in antigenic structure, and, if not alike, whether the differences are qualitative or merely quantitative.

[1] Dissertation presented to the Faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy.

For convenience, only the results of agglutination tests will be presented in this paper, since they form an entity which may be considered and discussed apart from the more satisfactory and conclusive results obtained with the complement-fixation reaction.

The numerous reviews (Arkwright, 1914; 1921: Buchanan, 1919: Tulloch, 1915), previously published, of the factors affecting agglutination make still another summary of the literature superfluous; and references to the findings of other workers will be limited to those which have a direct bearing on the experimental data presented below.

EXPERIMENTAL DATA

For the study of the antigenic relationships of *Bact. typhosum* approximately 150 typhoid cultures were collected from various laboratories situated in all parts of this country, in Canada, and in the Canal Zone. This series may be considered representative of *Bact. typhosum* as found in North America.

It may be noted here that all strains have been identified in this laboratory as *Bact. typhosum* by their cultural, agglutination, and complement-fixation reactions, and in the case of strains from other laboratories, their identity has been ascertained by someone other than the writer. In no case has a strain been included as *Bact. typhosum* which gave reactions incompatible with its classification as such.

The simplest and most obvious method of determining possible antigenic differences is agglutination. Although the results cannot be accepted as final, owing to the occurrence of relatively or absolutely inagglutinable strains, still it was hoped that agglutination tests would serve to point out some differences between the strains and provide a clue to those strains which should be more carefully and thoroughly studied.

The agglutination experiments can be divided into two parts: Those done with living saline suspensions and those with formalinized broth cultures prepared by Dreyer's method.

I. Agglutination tests with saline suspensions

For these experiments the organisms were grown on extract agar slants for twenty-four hours, and washed off with sufficient sterile physiological saline solution to give suspensions of appropriate and approximately equal density. Serum dilutions were made in glass bottles and 0.5 cc. quantities distributed from these stock dilutions to small tubes, to which 0.5 cc. quantities of the suspensions were then added. The tests were incubated for two hours in the 37°C. water-bath and then placed in the ice-box. Readings were made the following morning. In this way all the available cultures were tested, lots of 10 to 40 strains being done at one time.

It is unnecessary to give in detail the results obtained with all strains, for a single protocol will provide the salient features. From the examination of such a protocol (table 1; sera EU, EW, Rawlings) it is obvious that some strains are agglutinated more strongly than others when tested with any one serum; that the readily and poorly agglutinable strains, as determined by their reaction with one serum, do not necessarily coincide with a similar classification based on the results with another serum; and that some strains are not agglutinated in significant dilutions by any of the sera used. For example, strain W was agglutinated in dilutions of 1:3200, 1:800, and 1:400 by sera EU, EW, and Rawlings, respectively; strain U in dilutions of 1:400, 1:400, and 1:1600; and strain A in dilutions of 1:400, 1:800, and 1:400. Strain S was not agglutinated by any of the three sera in dilutions as low as 1:100. With serum EU, then, these strains would arrange themselves W > U and A > S, in order of agglutinability; with serum EW, W and A > U > S; and with serum Rawlings, U > W and A > S. Entirely similar phenomena were found in the other experiments.

That these preliminary findings could not be unconditionally accepted as demonstrating antigenic variation was shown in an experiment in which a number of Rawlings strains were tested. The Rawlings strain, according to Nichols (1915), was isolated in 1900, in England, from the spleen of a fatal case of typhoid

TABLE 1

Agglutination tests with saline suspensions

STRAIN	SERA			
	EU	EW	Rawlings	V
A	400	800	400	
C	400	400	200	
D	400	200	400	
E	200	200	400	
F	200	400	200	
G	400	400	−100	
H	400	400	100	
K	200	200	100	
L	400	400	400	
M	800	400	100	
N	800	400	800	
O	800	800	800	
P	800	800	800	
R	100	−100	400	
S	−100	−100	−100	400
T	800	800	1600	
U	400	400	1600	
V	100	100	200	
W	3200	800	400	
X	−100	100	−100	800

The figures gives the highest dilution in which complete agglutination occurred. A figure preceded by a minus sign (−) signifies that complete agglutination was not present in this and higher dilutions.

TABLE 2

Agglutination tests with formalinized suspensions

ANTIGENS	SERA								
	FK	AV	R	Rawlings	DW	EW	CH	EU	V
R	114	90	100	100	25	40	45	20	88
V	114	80	90	100	100	20	33	40	100
AV		20							
CH	128	110	60	75	50	60	100		25
DW	114	70	30	75	100	80	88	40	
ET	128	110	60	100	125	60	76	40	100
EW	128	100	100	25	100	100	112	80	112

The figures represent agglutination in percentage of the titre of the serum for its homologous strain.

Where no figure is given, complete agglutination did not occur in any dilution tested.

fever. A transplant of this culture was received by the Army Medical School at Washington in 1908, and has since been widely distributed in this country. In response to our request for

TABLE 3

Agglutination tests with Rawlings and Hopkins strains

STRAIN	SERA									
	EU	EW	Rawlings	FK	Sm	La	Ro	Ba	Lo	Li
D	800	800	400	400	−20	−20	−20	−20	−20	640
L	800	400	800	1600	−20	80	80	−20	160	1280
M	−100	−100	−100	3200						1280
Y	400	−100	−100	800	−20	−20	−20	−20	160	160
AB	400	400	400	3200	20	40	80	−20	160	1280
AC	200	400	200	3200	20	−20	80	80	160	640
AE	−100	−100	400	−200	−20	40	−40	20	160	Spont.*
AN	400	800	400	800	−20	−20	−20	−20	−20	−40
AR	800	400	800	6400	−20	80	−20	20	−20	1280
BC	400	400	800	−200	20	40	−20	160	−20	Spont.
BY	800	400	−100	1600	−20	160	−20	−20	−20	−40
CD	400	400	400	6400	−20	160	160		160	1280
CS	800	800	400	1600	−20	−20	80	−20	160	640
CW	800	800	400	6400	−20	−20	40	20	160	640
DN	200	400	800	3200	−20	−20	80	160	160	1280
EA	400	1600	200	1600	20	−20	80	160	160	1280
ET	400	800	800		−20	160	−20	160	160	Spont.
N	800	1600	800		−20	−20	−20	40	80	
AA	400	800	800		−20	−20	−20	40	20	
AD	1600	800	400		−20	−20	80	20	−20	
AF	1600	3200	800		40	20	−20	−20	40	
AL	1600	1600	800		20	−20	20	−20	80	
AY	1600	800	800		−20	−20	−20	20	−20	
BF	200	200	800		−20	−20	−20	160	−20	
CN	−100	100	−100		−20	−20	20	20	−20	
ES	800	800	400		−20	−20	−20	40	−20	

Rawlings strains: first 17 (D to ET).

Hopkins strains: last 9 (N to ES).

Monovalent rabbit sera: EU, EW, Rawl., FK.

Human sera (inoculated): Sm, La, Ro.

Human sera (patients): Ba, Lo, Li.

Figures are dilution in which complete agglutination occurred; i.e., 800 = complete agglutination in serum diluted 1:800.

Figures preceded by minus sign indicate incomplete or no agglutination in this and higher dilutions.

*Spont., Spontaneous agglutination.

typhoid cultures we received seventeen Rawlings strains from sixteen different laboratories. These were tested for agglutinability with the three sera used in the previous experiments and also with two sera from typhoid patients and three from prophylactically inoculated normal persons (table 3). At the same time a number of cultures of the Hopkins strain were similarly examined.

Recently, twenty months after the original experiments, the Rawlings strains have been again tested with two sera (table 3, Sera FK and Li). One of these is a rabbit serum prepared with a recently isolated strain (FK) and the other was obtained from a patient. Differences similar in character to those found in the earlier experiments are still noticeable in the later ones, in spite of the fact that for over a year and a half the environment of all the cultures has been the same.

II. Agglutination tests with formalinized broth suspensions

The marked differences in agglutinability noted in the preceding series of tests can not be accepted as conclusive evidence of variation, in view of the numerous sources of error inherent in the agglutination method and of the variable reactions disclosed by the several Rawlings strains. Although, then, these findings are not valid evidence of *definite* antigenic difference, they may be accepted as evidence of *possible* antigenic difference, and it is from this standpoint that they are considered to possess some value.

A number of strains which apparently differed more or less widely from each other in the experiments described above were selected and formalinized broth suspensions were made of them after they had been transplanted in infusion broth daily for two weeks (Dreyer, 1919). Nineteen such suspensions were made, each from a different strain.

The tests were carried out in the same way as the preceding series, except that incubation was done at 55°C. and that a change was made in the method of diluting the serum. Instead of using dilutions which proceeded by geometrical progression, the dilu-

tions progressed arithmetically, the interval between successive dilutions representing about 10 per cent of the titer of the serum for its homologous strain. This procedure possesses two advantages: First, the higher dilutions lie closer together, which tends to bring out slight differences between strains which are strongly agglutinated; and second, results with different sera can be readily compared by charting agglutination in terms of the percentage of the serum's titer rather than as serum dilutions.

In a few experiments all nineteen strains were tested, but later, as similarities appeared, this number was reduced to seven. Several more tests were conducted with these suspensions before it was decided to abandon the use of agglutination tests for the solution of the problem.

TABLE 4

Diagnostic agglutination tests with Rawlings and Hopkins strains

	POSITIVE		NEGATIVE	
	Rawlings	Hopkins	Rawlings	Hopkins
Ba	5	4	10	5
Lo	11	3	5	6
Li	12		2	

The reactions (table 2) obtained by this technic differ only slightly in detail, and not at all in character, from those obtained with the saline suspensions. With most of the sera several strains were agglutinated to titer; although, the particular strains which so react, vary with the serum used. Similarly, all strains (except AV) were strongly agglutinated by one or more sera. This observation is of importance, since it proves that failure of a suspension to be agglutinated was not due to inherent inagglutinability.

DISCUSSION

In the experimental work reported above, a number of typhoid strains have been tested for agglutinability with a number of monovalent anti-typhoid rabbit sera and with several human sera. The strains employed have differed in source, age, and environment since isolation. A preliminary series of experiments

was performed in which saline suspensions of the organisms were tested, in groups of 10 to 40 strains, for agglutinability with three sera. After all the available cultures had been thus examined, nineteen strains were selected as representative of the collection. These were used in the preparation of formalinized broth suspensions by Dreyer's method, and then tested for agglutinability.

The outstanding feature of all the experiments is the failure of the strains to react alike. While nearly all the strains examined were well agglutinated by one or another serum, yet it is apparent that no one monovalent serum would serve to identify them all as *Bact. typhosum*. In other words, the failure of a strain to agglutinate with a given serum must in most instances be attributed to lack of appropriate agglutinin in the serum rather than to inherent inagglutinability of the bacteria. This is particularly true if formalinized broth suspensions are employed, for all but one of these were agglutinated to full titer by more than one serum.

Inagglutinability, either absolute or relative, of typhoid cultures has been attributed to various factors. Among these are lack of motility, insufficient transplantation since isolation, recent contact with immune serum, tendency to spontaneous sedimentation and agglutination, contamination by the bacteriophage of d'Herelle, or the use of serum of the wrong antigenic group. In the present investigation all but the last named can be disregarded since the cultures used have been shown to be agglutinable, as was indicated in the preceding paragraph.

The one question left for discussion, then, is the possible presence of antigenic groups or at least of antigenic differences among typhoid strains. References may be cited either in favor of, or against, the presence of such groups. Dreyer, Gardner and Walker (1921) maintain that such differences as exist in the agglutinability of typhoid strains are merely quantitative, so that the relative agglutinability of any two or more strains may be expressed as a numerical ratio or "coefficient of agglutinability." Opposed to this view is the theory of antigenic groups as put forward by Weiss (1917), by Downs (1921), and by Hooker (1916-1917). Of these the first two have used agglutination and

agglutinin-absorption tests and the last has employed the complement-fixation reaction. Weiss found 7 and Downs 4 groups, while Hooker was content with only 3. This question will be left for fuller discussion in another paper, but it is apparent that the experimental results reported here do not support either view. Dreyer's opinion is flatly contradicted, and to this extent the proponents of the "group" theory are upheld. But, on the other hand, there is no conspicuous evidence of clear-cut antigenic types. From the standpoint of the agglutination reaction, the relationships of the different strains are so intricately complex that the possibility of classification appears remote. Even though it could be accomplished, it would have to be based on very fine and arbitrary distinctions.

The most interesting phase of the work is the series of tests conducted with the Rawlings strains. All of the strains have been tested with at least nine sera. Some of the tests were performed shortly after the cultures were received, the others after the lapse of twenty months, during which time all strains were maintained in the same environment. We must assume that the cultures sent to us as Rawlings strains are really descendants of the original strain and have not been erroneously labelled as such. Taking it for granted that our assumption is correct, the evidence is definite and clear-cut that changes have taken place, and that Rawlings strain in laboratory "A" is not identical in its reaction to agglutinating sera with the Rawlings strain in laboratory "B."

It is felt that the differences brought out in these experiments may serve to explain some of the discrepancies noted when Widal reactions are reported from two or more laboratories. In table 4 are tabulated the results obtained when the sera of different patients were tested against the seventeen Rawlings and Hopkins strains. Complete agglutination in a dilution of 1:40 is regarded as a positive reaction, and no agglutination (at 1:40) as negative. The results with the serum last tested show more uniformity but even this serum failed to give complete agglutination with two of the strains, despite the fact that its titer was above 1:1280. The presence of such marked differences in cultures derived from supposedly the same source is proof of the fallibility of a method of grouping based upon the agglutination reaction.

CONCLUSION

1. Agglutination tests of a number of typhoid strains show that such strains present considerable antigenic differences in so far as such tests furnish valid evidence.

2. These differences are not sufficiently clear-cut and definite to permit the designation of types or groups; particularly since the descendants of a single culture may show similar variations.

3. The failure of a strain to be agglutinated by an anti-typhoid serum is not proof of the non-typhoid nature of the strain; nor is failure of a serum to agglutinate a strain of *Bact. typhosum* proof of a lack of typhoid agglutinin in the serum.

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THE RELATION OF DIGESTIVE ENZYMES AND FERMENTS TO THE PHENOMENON OF D'HERELLE

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Received for publication February 24, 1923

Since the French edition of "The Bacteriophage" by d'Herelle was published in October, 1921, and the English edition in March, 1922, it hardly seems necessary to preface this discussion with the customary explanatory remarks concerning the phenomenon of bacteriophagy.

It will be recalled that in d'Herelle's discussion on the nature of the bacteriophage, three hypotheses are submitted. First, that "the bacteriophage is derived from the superior organism in its reaction to the bacterial invasion by the production of a principle which provokes the destruction of the bacterium." Two possibilities, he says, are admissible under this hypothesis. (1) That the principle mentioned is of a diastatic nature. (2) That the principle is particulate, an organic being, capable of developing outside of the organism at the expense of the bacteria. Second, that "the bacteriophage may be derived from the lysed bacterium itself." According to Kabeshima (1920a and b) the bacteria secrete a diastase with autolytic functions. Bordet and Cuica (1920, 1921, 1922) have sponsored a combination of these first two hypotheses as a probable explanation. Third, that "the bacteriophage is an autonomous organism, an ultramicrobe parasitizing the bacteria." The latter view is that held by d'Herelle.

We have concerned ourselves in this work with a study of various digestive enzymes and ferments with the idea of determining what, if any, relation exists between these substances and

the phenomenon of bacteriophagy. Davison (1922) in a recent article concludes that the lytic principle in d'Herelle's phenomenon is possibly an enzyme. Working with one and five per cent solutions of trypsin in normal saline and twenty per cent alcohol he showed that there was no bacteriolytic activity of these solutions against cultures of normal dysentery bacilli, although his solutions were chemically active. Combiesco (1922) working with enterokinase and trypsin, treating each with alcohol for periods varying from twenty-four to seventy-two hours and heating at 55°C. and from 60 to 70°C., reports the presence of a transmissible lytic principle except where the higher temperatures were used. He failed to demonstrate any lytic activity with papain after 40 passages. He states that the activity found in enterokinase is much stronger than that found in trypsin. Pico (1922) claims to have demonstrated the lytic principle in solutions of pancreatin, trypsin, papain and papayotin (carase, papain or papase), after heating to 100°C. Bachman and Aquino (1922) have reported the presence of a lytic principle in pancreatin. Kuttner (1923) reports the presence of a lytic principle in 25 and 50 per cent glycerol extractions of the small intestines of guinea pigs and acetone extractions of the liver tissue. We have work now in progress which bids fair to confirm these findings.

Desiring to cover the field as fully as possible it was necessary to go to the open market and obtain enzymes prepared for biologic purposes. In each case we have included the dealer or manufacturer's name and such data regarding the preparation as might be of interest.

In testing the bacteriolytic activity of Diastase of malt (Eimer and Amend), 10 per cent solutions in water having pH values of 6.9 and 7.4 respectively were used. With each enzyme tested the optimum pH for chemical activity as well as for bacteriolysis was used. In this experiment 1 per cent pepton water (pH 8.0) bacterial suspensions were prepared by adding one drop of a twenty-four hour broth culture of the organism to each tube and these were then incubated for twelve hours. The enzymes were placed in solution and the hydrogen ion concentration determined, after which these solutions were filtered through a Mand-

ler No. 4 candle, incubated and cultured to prove their sterility. To 8 tubes containing twelve hour bacterial suspensions of Shiga and Flexner dysentery bacilli (4 tubes in each set), were added amounts of a 10 per cent water solution of the enzyme varying from 0.5 to 1 cc. To the same number of tubes of bacterial suspension were added similar amounts of the enzyme solution of the higher pH concentration. Control bacterial suspensions and medium were of course included in the series, the whole being placed in the incubator at 37.5°C. Chemical activity of the enzyme solutions was then determined with the material remaining and both solutions were found to be markedly active chemically when tested with starch. Readings were taken at periods of twelve hours up to seventy-two hours. No macroscopic bacteriolytic activity was evident during this period of observation. Upon plate subcultures there were found to be no "sensitive" colonies after twenty hour hours nor were we able to demonstrate any "colonies" of bacteriophages upon agar slant subcultures from the various tubes. These results were uniformly obtained after repeated passages.

In testing the bacteriolytic activity of Taka-Diastase (Parke, Davis & Co.), claimed by them to liquefy 300 times its weight of starch in ten minutes, sample 2526314, a 10 per cent solution in 0.85 per cent saline of pH 7.5 was used. Bacterial suspensions of Shiga dysentery bacilli were prepared as in the preceding experiment. The enzyme solution was filtered, incubated and tested for sterility as described above. The test was then applied in a like manner. The material was markedly active chemically when tested with starch solution. Readings taken at twelve hour intervals during incubation up to seventy-two hours showed no evidence of bacteriolytic activity. There were no "sensitive" colonies or colonies of bacteriophages demonstrated in subculture. A known bacteriolysant from our stock used as a control gave the characteristic findings.

In the study of Pepsin, two different commercial preparations were used. Parke, Davis & Co. powdered pepsin, U.S.P. marked 1:3000, sample 2550561 and Eli Lilly & Co. granular pepsin, U.S.P. The difficulty attending the study of this enzyme in

connection with this problem is manifestly one of reaction. For this reason the enzyme was tested under a variety of conditions, varying reaction, strength of enzyme in solution, subsequent dilutions with bacterial suspensions and medium employed. Both preparations of pepsin were used in five and ten per cent water solutions. Bacterial suspensions of normal Shiga dysentery bacilli were prepared and tested as in the preceding experiments. The hydrogen ion concentration of the pepsin solutions was varied from pH 6.4 to 7.4. In some cases sodium hydroxide was used to neutralize the acid present (in which case the chemical activity of the pepsin was destroyed) and in other samples we made use of calcium carbonate followed by weak alkali such as sodium acetate or milk of lime which was added very cautiously. Solutions of pepsin made neutral or slightly alkaline in this manner do not lose their chemical activity and can be reactivated upon the addition of small amounts of hydrochloric acid. Untreated samples of pepsin in solution, pepsin solutions to which as much as 0.3 per cent hydrochloric acid were added and samples which were first neutralized as described above and then made slightly acid gave macroscopic lysis in the tubes of bacterial suspensions. Upon plate and agar subcultures however, there were found to be no "sensitive" colonies or colonies of bacteriophages. The time in which lysis took place varied directly with the acidity of the solutions. We are inclined to the view that where "clearing" took place, it was more a question of reaction and addition of inhibitory substances (acid) than of lytic activity of the pepsin. Indeed a subsequent study of the effect of different normalities of hydrochloric acid and sodium hydroxide when 0.5 cc. amounts were added to bacterial suspensions of Shiga dysentery bacilli showed that N/5 hydrochloric acid would cause "clearing" in the tubes after twenty-four hours incubation while the N/5 sodium hydroxide would not clear all the tubes in the series until forty-eight hours had passed. Similarly N/10 hydrochloric acid caused "clearing" in all the tubes in seventy-two hours while sodium hydroxide of this concentration had no effect when added in the same quantity.

Davison stated that N/5 sodium hydroxide would check the action of a bacteriophage; that N/1 sodium hydroxide killed dysentery bacilli and also lysed them; that 20 per cent hydrochloric acid killed the cultures but did not lyse them. We found as did he, that the organisms treated with hydrochloric acid stained with more difficulty.

It is apparent then that bacterial suspensions of Shiga dysentery are more sensitive to hydrochloric acid than to sodium hydroxide and so for this added reason we feel that the "clearing" of the bacterial suspensions with addition of acid pepsin solutions was due to the acid present and not to the presence of any lytic activity of the enzyme.

To further substantiate this view the effect of acid pepsin and alkaline pepsin on living and dead (killed by heating to 80°C. for twenty minutes) bacterial suspensions of both Shiga and Flexner dysentery bacilli was tested by making bacterial counts. Stained smears from cultures were prepared both before and after incubation with the enzyme solutions to determine if the organisms were lysed. Subcultures were made before and after incubation to determine the presence of living organisms. These were found to be negative after incubation. The bacterial counts of the Flexner and Shiga dysentery suspensions at the beginning of the experiment (twelve hour cultures) were 20,400,000 and 14,320,000 respectively. After twenty-four hours incubation with the acid pepsin solution the counts were 9,440,000 and 8,750,000 while after incubation for twenty-four hours with the alkaline pepsin solution the counts were 15,200,000 and 9,540,000 respectively. The stained smears before incubation in each case were graded plus-minus in regard to the amount of debris present. After incubation for twenty-four hours with the pepsin solutions the stained smears were graded two plus in each case. The killed cultures of Flexner and Shiga dysentery at the beginning of the experiment gave counts of 19,200,000 and 15,400,000 respectively. After twenty-four hours incubation with acid pepsin the counts were 4,320,000 and 6,020,000 while after incubation with the alkaline pepsin the counts were 5,440,000 and 8,360,000. The stained smears at the beginning

of the experiment were graded one plus in each case while after incubation with the acid and alkaline pepsin solutions the stained smears were recorded four plus in regard to the amount of debris present. The acid pepsin solution was chemically active while the alkaline pepsin was not.

A commercial preparation of Pancreatin was obtained from Parke, Davis & Co. One per cent solutions of this preparation had a marked chemical activity upon fat and carbohydrates but its tryptic activity was only slight. We have been unable to obtain a satisfactory preparation of trypsin as such, though we believe these results with pancreatin substantiate those of Davison with regard to this particular enzyme. Five and 10 per cent solutions in physiological saline and also in 20 per cent alcohol were tested. The absence of any bacteriolytic activity in any of the solutions was uniformly definite. There were no "sensitive" colonies or colonies of bacteriophages on subcultures after repeated passages. A preparation of Metagen, which is claimed by Parke, Davis & Co. to be a combination of fat soluble A, water soluble B and C vitamines was tested in this same series and found to possess no bacteriolytic qualities nor did it aid the growth of the suspensions when compared with the control suspensions. Pancreatin was tested against bacterial suspensions of Flexner dysentery, *Bact. coli* and *Bact. typhosum* with the same negative results as with Shiga dysentery.

While the digestive function of the pancreas is of much importance and has been well understood for some time through the chemical study of this preparation called pancreatin, investigators have long thought there was also another most important secretion from this gland which until recently could not be isolated. We refer to the preparation known as "insulin" or the active secretion from the Islands of Langerhans of the pancreas. In connection with a study of methods of preparing "insulin," having obtained a very active preparation, 350 mgm. of the powder being capable of reducing the blood sugar in rabbits as low as 0.04 per cent and producing convulsions or death, we thought it would be worth while to test its bacteriolytic activity. Fourtenths of a gram of "insulin" was dissolved in 20 cc. of sterile

physiological salt solution. It was then filtered through a Mandler No. 4 candle and a subculture agar slant made to test its sterility. Bacterial suspensions of *Bact. coli*, *Bact. typhosum*, Parathphoid A and B, Shiga and Flexner dysentery were prepared having a series of 3 tubes for each organism. The solution of "insulin" was then added according to the d'Herelle technique for testing filtrates, i.e., 2 drops in the first tube, 10 drops in the second and 2 cc. in the third. Controls were of course included in the series. It should be stated that this preparation of "insulin" was very slightly acid to litmus. The entire series was placed in the incubator and observations made every twelve hours. There was no evidence of any lytic activity in any of the tubes nor were there any "sensitive" colonies or colonies of bacteriophages upon subsequent subcultures. These results obtained after repeated passages.

A commercial preparation of Papain (Merck, sample 12469-LS 55821), was then tested as heretofore described against twelve hour bacterial suspensions of both Shiga and Flexner dysentery bacilli. This preparation was an old one but yet fairly active chemically. There was a uniform absence of any bacteriolytic activity after repeated passages. We may mention that experiments with the bile salts have also repeatedly given us these same negative results.

SUMMARY

Commercial preparations of various enzymes including diastase of malt, Taka-diastase, pepsin, pancreatin and papain were tested for bacteriolytic activity against twelve hour bacterial suspensions of Shiga dysentery and in some cases against Flexner dysentery, *Bact. coli* and *Bact. typhosum*. All of these preparations were active chemically. The enzymes were tested under a variety of conditions simulating the optimum conditions for bacteriolysis and chemical activity. In no case, except with pepsin, was there any suggestion of bacteriolytic activity. We believe the action present in solutions of pepsin is due to the acid reaction and not to the activity of the pepsin. A preparation of "insulin" which is active chemically by the biologic test was tested for its

bacteriolytic activity against members of the colon, typhoid and dysentery group of organisms. Solutions of the bile salts and a solution of Metagen (vitamines) have been likewise studied.

CONCLUSIONS

If the lytic principle in d'Herelle's phenomenon is an enzyme, this enzyme is not diastase of malt, Taka-diastase, pepsin, a combination of trypsin, steapsin and amylopsin as found in pancreatin or a combination of a proteolytic and amylolytic enzyme as found in papain. The lytic principle is not "insulin," the active secretion of the Islands of Langerhans of the pancreas. It is not related to the bile salts or to vitamins in so far as this can be determined by commercial preparations which are available. If the diastatic theory as to d'Herelle's lytic principle is to prove tenable more information of a chemical nature must be brought to bear upon the problem.

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AN APPARATUS FOR TUBING SEMI-SOLID MEDIA

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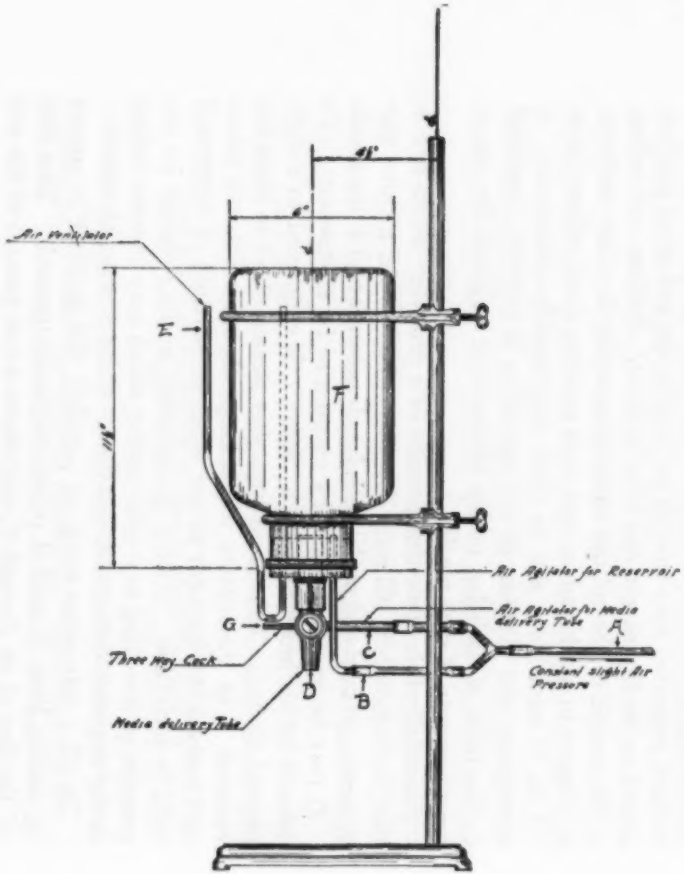
Received for publication April 6, 1923

We have had considerable difficulty in finding a satisfactory method for tubing semi-solid media, such as those containing egg, meat or brain. Upon inquiry of other workers, we find they experience the same difficulties. Methods commonly in use are cumbersome, mussy, and require the use of large tubes. The method presented here enables us to tube egg and meat medium almost as readily as one can tube bouillon. Spilling, and soiling of the mouth of the culture tube, so annoying in other methods, is reduced to a mimimum, and even medium sized culture tubes (16 to 17 mm. bore) can be filled very rapidly and neatly. A description and diagram of our apparatus is herewith appended in the hope of assisting others whose work requires the use of large amounts of such media.

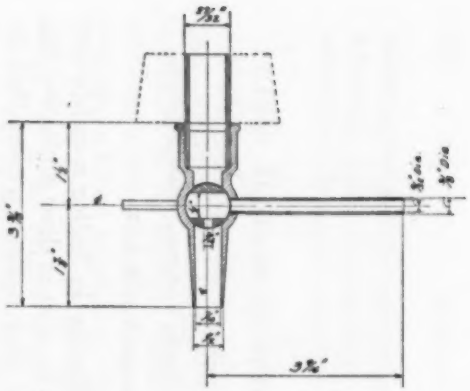
Essentially this apparatus consists of a reservoir, which is a 3-liter, wide-mouthed (65 mm. diameter) glass bottle, a three way stopcock, an inlet and outlet tube for air pressure and a ring stand.

It may be operated either by vacuum or by air pressure. To operate by air pressure, tube *A*, figure 1, is connected to a compressed air line. Tube *B* allows a slow stream of air to enter the reservoir *F* at the bottom which keeps the contents well mixed and enables the operator to get equal proportions of liquid and solid in each tube. Tube *C* is connected to the stopcock for the purpose of removing any solid matter which may become lodged in the stopcock and also for agitation. Tube *E* is the air outlet.

To fill a tube,when using air pressure, the stopcock is opened by raising lever *G* until it points vertically upwards. This cuts off the flow of air through *C*, and allows a free passage of the well



SIDE ELEVATION Scale ½"=1"



SECTION THROUGH THREE-WAY COCK
Scale Full Size.

APPARATUS FOR FILLING SEMI SOLID MEDIA

FIG. 1

mixed medium into the culture tube. The stopcock is then closed by lowering the lever to a horizontal position pointing away from the operator which reëstablishes a flow of air through *C*. Solid medium is thus dislodged from the upper half of stopcock and again set in agitation. If any solid material should become lodged

FIG. 2

in the lower half of the stopcock, it may readily be discharged by turning the cock so that the lever is pointing horizontally toward the operator. This enables the air coming through tube *C* to pass through the lower half of the stopcock and out at the nozzle *D*.

The photograph, figure 2, shows the appearance of the apparatus when operated by vacuum.

To operate with vacuum, tubes *A*, *B* and *C*, figure 1, are removed entirely. The hole where tube *B* enters the reservoir is stoppered by inserting a glass rod. Tube *E* is connected to a vacuum line. The air enters through tube *C* keeping the stopcock clear and keeping the contents of the reservoir *F* well mixed. The stopcock is manipulated the same as when tubing by air pressure.

THE ATTENUATION OF BACTERIA DUE TO TEMPERATURE SHOCK[1]

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Received for publication February 14, 1923

I. INTRODUCTION

The phenomenon of attenuation of bacteria is so closely related to that of stimulation that it seems almost impossible to deal with one without making considerable reference to the other. Within certain limits it may be true that a stimulus for one bacterial faculty, may at the same time attenuate the organism in some other manner before it fails as a stimulant. Heat sometimes alters an organism in such a way that one function may be attenuated while others are not. Pasteur, according to Fisher found that heat may cause the Anthrax organism to lose its virulence while at the same time not losing its ability to grow and to multiply.

Before the discussion of attenuation of bacteria is carried further it is important that the term be defined so as to prevent misunderstanding. The word attenuation is derived from attenuis, to make thin. As used, attenuation means the lessening of one or more functions of bacteria due to abnormal conditions, temporary or prolonged. A wider use of the term was at first considered, that is, the lessening of all of the functions of an organism. However this wide meaning of the term was found untenable due to the fact that our knowledge of bacteria is largely gained by observations of their separate functions. Attenuation is used as an opposite of the term stimulation in the following discussion and

[1] This is a résumé of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in bacteriology in the Graduate School of the University of Illinois, 1922.

refers to one or more bacterial functions and not to the total of bacterial functions. Thus as far as the use of the term goes, a culture of bacteria may be attenuated as to one function and at the same time be stimulated in other respects.

The subject of the attenuation of bacteria divides itself into three important fields of study: (*a*) Attenuation of virulence, a field of science which has revolutionized medical practice; (*b*) attenuation and stimulation of functions of bacteria for commercial advantages, a field of study promising wide application in the manufacturing industries; (*c*) attenuation of the normal, physiological functions of bacteria, phenomena which are rapidly gaining attention because of their relation to the problems of evolution.

There is no intention here to go into the subject of the attenuation of microörganisms as used in producing immunity in living things but simply to mention how thoroughly grounded it is and of how great service to man it has been. Considering the remarkable results obtained in this field it seems rational to believe that other bacterial functions than that of virulence might be manipulated and certain advantages obtained thereby.

There is more or less material to indicate that some of the functions of microörganisms may be altered for definite periods of time. Takamine, in the manufacture of diastase altered *Aspergillus oryzae*. Adams speaks of the modification of yeast cultures in glycerol manufacture. Wehmer reports the loss of oxalic acid producing power by *Aspergillus niger*. Euler and Svanberg speak of temperature adaptation of yeast. LaFevre and Round suggest that certain organisms may acquire salt tolerance and perhaps later require a certain amount of salt for best development. Zikes reports that he changed the generation time of certain yeasts by prolonged cultivation at abnormally low temperatures.

II. PURPOSE OF THIS WORK

It was the general purpose of the following investigation to gather further data as to the possibility of suppressing the bacterial function of multiplication. While it has been generally considered that the increased keeping quality of pasteurized milk

is due entirely to reduction in numbers of bacteria, still the question may be raised whether a part of this keeping quality might not be due to the attenuation of bacteria during the process of pasteurization. It is the object of the following work to measure if possible any change in the generation time of certain bacteria of market milk before and after the temperature shock of pasteurization.

III. METHODS OF STUDY

Thirty-eight samples of raw market milk were plated on one per cent lactose agar and from these plates were selected 144 predominating colonies. These organisms were then added to litmus milk and incubated for fifteen days at 20°C. From litmus milk cultures there were selected at the end of fifteen days, nine representative organisms taking into consideration their action on litmus milk.

Each of the above nine cultures was then taken separately and comparisons made as to the effects of exposure to a temperature of 145°C. for thirty minutes on its rate of multiplication in sterile milk. Table 1 gives the result of comparisons of the generation times of each organism before and after the temperature shock.

To measure properly any possible attenuation which might occur in bacteria subjected to a heat shock, it is necessary to take into consideration certain variable influences in a milk medium which are more or less unfavorable to bacterial development. It has been the belief of some bacteriologists that milk contains antibodies similar to those in blood.

In order to eliminate the many complex factors involved in the use of fresh milk as a medium for the growth of organisms in this study, only separated milk which had been sterilized in the autoclave for one hour at 15 pounds pressure was used. Large amounts of this milk were sterilized at one time so that at least half of the comparisons of generation time of bacteria reported here were made in different small portions of the same batch of milk, all of which was sterilized at one running of the autoclave. These flasks of sterile milk were allowed to stand at 37°C. for several days to make sure of sterility.

TABLE 1

Effect of temperature shock upon bacterial increase

HOURS	UNTREATED BACTERIA PER CUBIC CENTIMETER	INCREASE	HEAT-TREATED BACTERIA PER CUBIC CENTIMETER	INCREASE
Series 8. *B. coli communis* No. 222.1113033 held at 20°C.				
0	1,550		518	
8	7,460	4.8	1,470	2.8
16	55,100	7.3	3,940	2.6
24	337,000	6.1	17,800	4.5
32	2,850,000	8.4	69,000	3.8
40	30,700,000	10.7	230,000	3.3
48	Coagulation		1,090,000	4.7
56			4,100,000	3.7
64			16,600,000	4.0
72			67,000,000	4.0
80			221,000,000	3.2
88			Coagulation	
Series 9. *Bact. lactis acidi* (B) No. 222.2223034 held at 20°C.				
0	26,700		22	
8	280,000	10.4	168	7.6
16	4,190,000	14.9	890	5.2
24	57,100,000	13.6	7,800	8.7
32	586,000,000	10.2	61,000	7.8
40	Coagulation		508,000	8.3
48			3,200,000	6.2
56			32,500,000	10.1
64			257,000,000	7.9
72			Coagulation	
Series 10. *Bact. lactis acidi* (B) No. 222.2223034 held at 20°C.				
0	54,000		1,000	
8	716,000	13.2	7,000	7.1
16	6,740,000	9.4	18,900	2.6
24	61,200,000	9.0	91,000	4.8
32	Coagulation		538,000	5.9
40			3,580,000	6.6
48			29,700,000	8.2
56			182,000,000	6.1
64			659,000,000	3.6
72			Coagulation	
Series 11. Micrococcus No. 222.3333623 held at 20°C.				
0	83		7	
2	98	1.18	6	0
4	212	2.16	11	1.83
6	330	1.55	25	2.27
8	502	1.51	55	2.20
10	940	1.87	82	1.49
12	1,130	1.20	137	1.67

To each of the two flasks of sterile milk in series 9 and 10, there was added 1 cc. of pure culture from a twenty-four hour growth in sterile milk. Then one flask of each series was left raw and one was pasteurized. These two flasks were then brought to the same temperature and agar plates made at the end of various periods as shown in table 1. One per cent lactose agar was used and colonies were counted at the end of two days at 37°C. and three days at 20°C.

In the case of series 8 and 11, the attempt was made to start each flask of a series at as nearly as possible the same number of bacteria per cubic centimeter. This was done by counting the number of bacteria in the milk culture and then calculating how much it would require of the pure milk culture to leave about equal numbers of bacteria per cubic centimeter after pasteurization of one of the duplicates. In order to arrive at this approximate number of bacteria per cubic centimeter, it was found necessary to determine the pasteurization reduction factor for each organism before starting to work with it.

The method of obtaining plate counts was as follows. Five different dilutions of each sample on 1 per cent lactose agar were made and plated in triplicate giving fifteen plates from which to obtain the bacterial count of the sample. In other words the seventeen determinations of numbers of bacteria showing progressive increase of bacteria in series 8 for example were obtained from 255 plates. The fourteen determinations in series 9 were obtained from 210 plates, and the same rule was carried through the determinations given in the other series. It was considered advisable to make what might be called excessive numbers of plates in order that the factor of variation due to the plate method might be reduced to its lowest point. Test tubes containing 9 cc. of sterile distilled water, were used for making dilutions. Vigorous shaking of dilutions was practiced. Care was taken that the 1 cc. of dilution was thoroughly mixed with the agar on each plate. Incubation of plates was for five days at 20°C. and two days at 37°C.

In determining the generation time the method used by Barber has been followed. If the number of bacteria at the beginning of

TABLE 2

Effect of temperature shock on generation times

UNTREATED BACTERIA						HEAT-TREATED BACTERIA					
No. G.	T.	G. T.		Ave. G. T.		No. G.	T.	G. T.		Ave. G. T.	
Data from series 8											
		hrs.	*min.*	*hrs.*	*min.*			*hrs.*	*min.*	*hrs.*	*min.*
2.20	8	3	37	2	54	1.41	8	5	40	4	30
2.84	8	2	48			1.34	8	5	57		
						2.12	8	3	46		
						1.93	8	4	8		
						1.66	8	4	48		
						2.18	8	3	39		
						1.88	8	4	15		
						2.01	8	3	58		
						2.00	8	4			
						1.64	8	4	52		
Data from series 9											
3.31	8	2	24	2	15	2.90	8	2	45	2	47
2.87	8	2	3			2.32	8	3	26		
						3.09	8	2	34		
						2.95	8	2	42		
						3.04	8	2	37		
						2.57	8	3	6		
						3.26	8	2	27		
						2.97	8	2	41		
Data from series 10											
3.65	8	2	11	2	25	2.77	8	2	52	3	36
3.17	8	2	31			1.33	8	6			
3.13	8	2	33			2.20	8	3	37		
						2.47	8	3	13		
						2.66	8	3			
						3.03	8	2	38		
						2.53	8	3	9		
						1.81	8	4	24		
Data from series 11											
0.18	2	11		5	25	0.00	2	0		2	7
1.08	2	1	51			0.83	2	2	8		
0.55	2	3	37			1.13	2	1	45		
0.52	2	3	50			1.10	2	1	48		
0.87	2	2	17			0.49	2	4	4		
0.20	2	10				0.67	2	2	58		

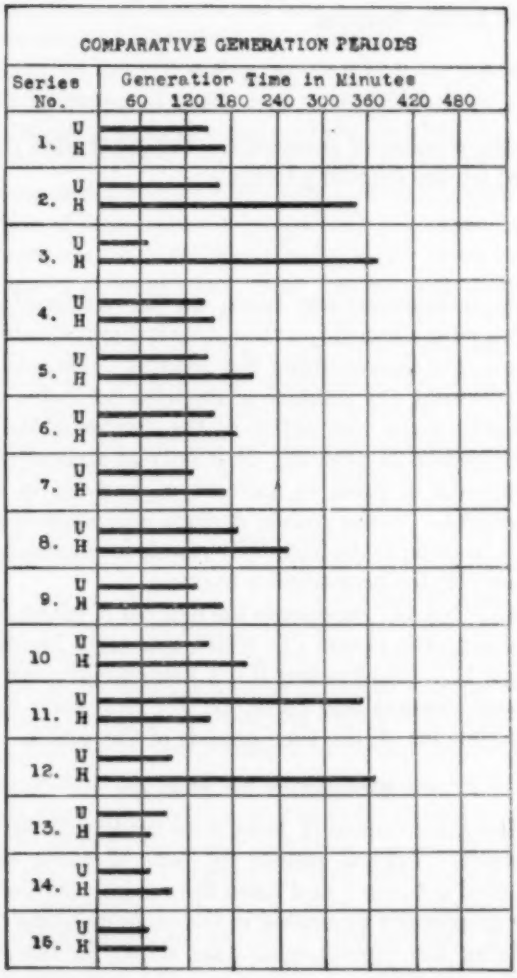
COMPARATIVE GENERATION PERIODS
Series No.
Generation Time in Minutes
60 120 180 240 300 360 420 480
1. U H
2. U H
3. U H
4. U H
5. U H
6. U H
7. U H
8. U H
9. U H
10 U H
11. U H
12. U H
13. U H
14. U H
15. U H

FIG. 1

a period of reproduction (a), and the number at the end of the period (b) be known, then the number of generations (n) can be determined by the formula:

$$2^n = \frac{b}{a}$$

Knowing the number of generations, the generation time can be determined by the following formula:

$$G = \frac{t}{n}$$

The above calculations are based on the assumption that all bacteria in a culure multiply at the same individual rate.

In table 1, the figures under the heading "Increase" were obtained by dividing the number of bacteria per cubic centimeter at the end of an eight hour period by the number at the beginning of the same period of growth. It is only of assistance in aiding the comparisons of rates of increase of bacteria of differently treated cultures. In the graphs showing comparative generation times given in table 1, the letter (U) stands for bacteria untreated by heat and (H) for heat-treated bacteria.

In table 2, "No. G." represents the number of generations which occurred during the period (T) which was eight hours in all the series except 11. The heading (G. T.) stands for generation time in hours and minutes and (Ave. G. T.) represents the average generation time for all the time periods of the series.

DISCUSSION OF RESULTS

Altogether fifteen series of tests were made with fifteen different organisms. Typical results for four of these series have been presented in tables 1 and 2 and the general result of the whole fifteen are graphically presented in the accompanying figure.

In studying the experimental data shown in the graph it is observed that in all the comparative series with the exception of nos. 11 and 13 the average generation time of the bacteria used, has been lengthened by the temperature shock of 145°F. for thirty minutes. Series 11 does not appear to be normal because

of the non-uniformity of the generation time at the beginning and at the end of the growth of the untreated bacteria. It is thought that other factors entered here due to the small number of bacteria per cc., and disturbed the comparisons.

In series 13, 14, and 15 shown on the graph the organisms used were spore bearers and there was no marked difference in the generation times of the untreated and the heat-treated organisms. The heat shock of 145°F. for thirty minutes is considered so far below the temperature exposure which kills the culture that comparisons were of little value in determining attenuation.

In the work with *B. subtilis* spores (series 13) more rapid multiplication was exhibited after than before heat treatment. It would seem that the heat shock of 145°F. for thirty minutes in some way caused stimulation rather than attenuation and it is probable that the temperature exposure is not high enough to threaten in any way the life forces of the spore.

From some of the literature concerning the heat resistance of spores it is evident that this is a very complex subject. H. Weiss reports that the spores of *B. botulinus* are normally destroyed in five hours at 100°C.; forty minutes at 105°C.; and six minutes at 120°C., and that spores one month old are three times as resistant as spores five months old. G. S. Burke found that the spores of *B. botulinus* are more highly resistant to heat when grown in brain cultures than in broth cultures. She reports that exposure to 100°C. or above attenuates the spores to such an extent that the incubation period of the spores is very much lengthened.

It seems very likely that different individual bacteria of a pure culture do not multiply at the same rate although we would expect the average generation time of several large groups of individuals of a pure culture to be quite constant. However, considering that when highly heat resistant bacteria are treated at the pasteurization temperature of 145°F., some individual bacteria die while some individuals are able to withstand even the temperature of boiling water, one would conclude that there is a marked difference between individual bacteria. When a pure culture of milk is treated to a considerable heat shock, the number of bacteria present is always more or less reduced. Those bacteria

which survive differ in some respect from those which die. If this difference were simply one of age of cells it might explain differences in multiplication rates of untreated and heat-treated cultures on the assumption that young and old cells do not multiply at the same rate.

Taking up this subject from another view point, it may be found that there are other characteristics than difference in age of cells which may accidentally accompany ability to survive a heat shock which may have something to do with the generation time of the cell.

To carry further the discussion of possible explanations of the differences in individual bacteria, considering our slight information of the subject would be mere speculation; but there are several additional factors which should not be overlooked. For instance, in two flasks containing pure cultures of the same organism in sterilized milk, when one culture has been subjected to a heat shock and has suffered a reduction in numbers of bacteria per cubic centimeter, the two cultures are no longer comparable. The two cultures differ by the fact that one has considerable numbers of dead cells present while the other culture has none or comparatively few. If we assume that the dead cells are a benefit to the living because of increased vitamine supply, or change in medium, we might conclude that this is a factor tending to shorten the generation time of the surviving bacteria of the culture treated to a heat shock. On the other hand, the rapid cooling of bacteria after a temperature shock is considered by Bushnell to be an attenuating influence in itself. Bushnell says.

> The influence of rapid cooling after heating is a matter of considerable importance in the canning industry. The only effect possible that this procedure could have would be that of shock to the bacterial cell. This might possibly devitalize the cell in such a way that it would be more easily destroyed by subsequent heating, or perhaps perish slowly, or not be able to grow under such rather unfavorable conditions as exist in the sealed container.

If it is a fact that the sudden cooling of heated bacteria causes a shock to the bacterial cells, this is an important factor in studying their rate of growth.

Another phenomenon occasionally referred to in bacteriological literature is the influence which numbers of bacteria inoculated into media have upon the growth of the culture.

It is reported by Churchman that when too few organisms are inoculated into certain media there is no growth, while a greater number of organisms produces growth. On the other hand we know that beyond a certain point, numbers of bacteria or their by-products check growth more or less.

Because of the many complex factors involved in growth, it is impossible to say definitely that attenuation is the cause of the different rates of growth of bacteria, heat-treated, and untreated.

VI. CONCLUSIONS

1. Assuming that the 15 organisms here used are representative of the flora of market milk and that these organisms would not relatively act differently in raw milk than in the sterilized milk here used, one seems warranted in drawing the conclusion that the lengthining of the period between production and souring of pasteurized market milk is not entirely due to the reduction in numbers of bacteria but is to some extent due to attenuation of bacteria during pasteurization.

2. Contamination of market milk after pasteurization is from the standpoint of keeping quality more important than the same amount and kind of contamination having survived the process of pasteurization.

3. In pasteurized milk some species of bacteria are killed outright, some are attenuated, and some are stimulated, depending on the resistance of the organism to heat. Those organisms which have a thermal death point below the pasteurizing temperature and time are of course killed off. Those organisms here tested which have a thermal death point a little above the temperature and time of pasteurization are attenuated markedly, while those which have a thermal death point far above the pasteurization time and temperature are attenuated very little or are stimulated to more rapid growth at least for a period.

4. In the development of methods of increasing the commercial life of some preserved food products, it seems very likely that

some benefit can be obtained by emphasizing the attenuation of microörganisms along with their partial destruction.

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THE MECHANICAL BEHAVIOR OF DYES, ESPECIALLY GENTIAN VIOLET, IN BACTERIOLOGICAL MEDIA

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Received for publication April 30, 1923

In studying the effects of the use of gentian violet in peptone enrichment media for water borne bacteria, a precipitation of the dye was noted after active growth of the bacteria in the broth. Several possibilities suggest themselves as possible explanations of this behavior. Such precipitation might for example be the result of the formation of a new compound —a decomposition product of the dye, or an absorption product, since no precipitation occurred so long as the broth remained sterile. Kendall and Bly (1922) in studying the changes of the nitrogenous constituents of ordinary media, found that there resulted, upon bacterial growth, an increase in the protein nitrogen fraction of the nitrogen content of the medium. They explained this by stating that an actual production of bacterial protein had taken place. T. B. Robertson (1908) says that the addition of proteins to watery solutions of dyes greatly alters the distribution of dyes between water and lipoid, and this alteration is characteristic for each protein. "Hence proteins combine chemically with certain dyes." Kligler (1918) states that the higher the concentration of organic nitrogenous compounds in the medium, the lower the effective concentration of the dye. Churchman (1921) has found that the addition of dead bacterial bodies to gentian violet media will permit organisms to grow which otherwise would not.

That the precipitation noted in the media was not due to reaction with the carbon dioxide or hydrogen, liberated during

bacterial growth, was shown by bubbling CO_2 gas through a broth containing gentian violet, 0.2 per cent lactose and 2 per cent peptone. Experiments were also tried with hydrogen gas both nascent and molecular. In no case was a precipitate obtained. In the presence of washed bacterial growth, molecular hydrogen, bubbled through a homogeneous gentian violet broth, completely decolorized the solution after one and a half hours, and a precipitate settled to the bottom. The hydrogen was not essential, however, as mechanical shaking of a gentian violet solution with washed bacterial protein yielded similar results.

The effect of time, hydrogen ion concentration and the presence or absence of a sugar in the medium, on the precipitation of dye was studied by the following experiment: Two sets of tubes were inoculated with seven cultures. The broth for the first set consisted of gentian violet 1 part to 200,000, 0.2 per cent lactose and 2 per cent peptone. That for the second set differed only in that it contained no sugar. The cultures used were of the colon-aerogenes group—all lactose fermenters. These cultures were incubated at 37°C. As would be expected, gas formed in all tubes containing lactose. In set I (lactose broth), after twenty-four hours only three of the tubes showed a greater amount of precipitation than the corresponding tubes of set II. After forty-eight hours, however, all tubes of the latter set showed a decidedly higher amount of precipitation. After five days incubation, the tubes of set II had become almost colorless, and complete precipitation had taken place, while in set I the maximum decolorization did not amount to over half the dye initially present. The apparent anomaly after only twenty-four hours incubation is to be expected since the presence of sugar would permit a more luxuriant growth during this period.

It took less than twenty-four hours to show the first degree of precipitation. Table 1 gives the values of the hydrogen ion concentration for all the tubes in Sorensen units.

With the exception of culture number 2, the limits of pH for the lactose broth are 4.3 to 4.8, and those for the broth contain-

ing peptone only are 8.0 to 8.5. The dye was taken from alkaline solution, therefore, rather than from the acid solution. In the case of number 2, the peptone broth was distinctly less alkaline than any of the others (pH 7.6) and not all of the dye

TABLE 1

NUMBER	BROTH	pH	PRECIPITATE	APPEARANCE OF LIQUID
2	Lactose	5.0	Light purple	Dark purple
	No lactose	7.6	Dark purple	Faint purple
3	Lactose	4.6	Light purple	Dark purple
	No lactose	8.4	Purplish black	Colorless
4	Lactose	4.65	Light purple	Dark purple
	No lactose	8.0	Purplish black	Colorless
16	Lactose	4.75	Light purple	Dark purple
	No lactose	8.5	Purplish black	Colorless
17	Lactose	4.8	Light purple	Dark purple
	No lactose	8.4	Purplish black	Colorless
19	Lactose	4.3	Light purple	Dark purple
	No lactose	8.3	Purplish black	Colorless
22	Lactose	4.4	Light purple	Dark purple
	No lactose	8.3	Purplish black	Colorless

TABLE 2

BROTH	pH	
	With sterilization	Without sterilization
Peptone	7.2	6.75
Peptone-lactose	6.8	6.5
Peptone-gentian violet	6.8	6.5
Peptone-lactose-gentian violet	6.8	6.5

was removed—though only a small amount remained. In the same instance the lactose broth showed less acidity than the other cultures, pH 5.0 and a little more of the dye had been removed from solution. The removal of dye, then, depends on

the pH of the solution and the above experiments suggest a theory of the effect of dyes on the growth of bacteria which is in line with Loeb's theory of protein behavior. It also leads to a theory of the mechanism of Gram stains, including the limitations of applicability and interpretation, on which work is at present in progress.

In connection with the above experiments, the results obtained were qualitatively confirmed by noting that the addition of small amounts of acid to cultures from which all the dye had been removed, caused it to be reliberated from the precipitate and distributed again throughout the solution, and also that further additions of alkali to this resulting solution again caused all the dye to be precipitated and the solution to become colorless.

Table 2 gives the values of the pH for the various broths used. Where peptone is stated it is as a 2 per cent solution, lactose is 0.2 per cent, and gentian violet 1 part to 200,000.

The pH of washed bacterial precipitate grown in a peptone-lactose broth, centrifuged and washed with distilled water was found to be about 7.6.

DISCUSSION

According to Loeb's theory, a protein is an amphoteric substance which combines with either acids or bases according to whether it is in a solution whose pH is respectively either less or greater than a certain value known as the isoelectric point of the protein in question. On the acid side of the isoelectric point the protein will combine with acids and on the alkaline side it will combine with bases. The isoelectric point of proteins is not necessarily the neutral point, in fact in the case of a large number of proteins whose isoelectric points have been determined all cases except that of gliadin show an acid solution and 75 per cent of those listed are at a pH of 5 or less (1922).

Now gentian violet is a basic dye, and if it combines with protein, this combination should take place on the alkaline side of the isoelectric point, and the amount of combination should increase with increasing alkalinity of solution and decrease with increasing acidity—which was demonstrated to be the case.

If the above theory is true, there should be a point at which the protein no longer takes up a basic dye such as gentian violet but as the acidity is increased it should combine more and more readily with an acid dye such as acid fuchsin. To test this out the following experiment was made: Buffer solutions were prepared between the pH limits of 2 and 5 in steps of about 0.3 of a pH unit. Approximately equal amounts of dead bacterial growth were introduced into tubes, equal volumes of the above buffers added and into each tube a certain quantity of gentian violet was introduced. The tubes were shaken and allowed to stand for several hours. As was expected, the color of the liquid gradually became lighter as the solution became more alkaline so that in the acid solutions very little of the dye was removed while in those solutions of higher pH more and more of the dye combined with the protein. It was found possible to wash out practically all the dye from the protein in the more acid solutions, and less and less of it as the pH increased.

A similar set of tubes was prepared and treated in the same way except that acid dyes were used. In this case the gradation of color after standing went the other way, i.e., more color was taken from the more acid solutions. Both acid fuchsin and ponceau were used and the results were parallel.

SUMMARY

Loeb's theory concerning the amphoteric character of proteins was demonstrated by the fact that the pH was found to regulate to a certain extent the amount of dye taken up by bacterial protein. A point was reached where the bacterial protein no longer took up a basic dye but as the acidity was increased it combined more and more readily with acid dyes. The basic dye used was gentian violet. The acid dyes used were acid fuchsin and ponceau.

Cultures are known to change from Gram-positive to Gram-negative with age. It is possible that this change may be brought about by treating the bacterial protein as growth pro-

ceeds with a solution whose pH is on the side of its isoelectric point opposite to that of the medium at the beginning.

It may also very well be that the effect of certain dyes in selectively inhibiting bacteria may find an explanation in considerations of the above nature.

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A SIMPLE MICROPIPETTE HOLDER

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Received for publication May 3, 1923

In the conduct of certain bacterial isolation work in this laboratory, it was found to be advantageous to use a micropipette; and a holder or manipulator was devised that proved very satisfactory. Since its construction was very simple as compared to others that have been described, it was thought that a description of the device would be valuable.

Barber (1914) is generally given credit for originating the micropipette method of isolating organisms and the method is frequently referred to as the Barber method. His apparatus was so complicated that it has not come into general use. Chambers (1923) recently described an improved device but from his description, the mechanism is not much simpler than Barber's original manipulator.

The device here discussed is very much simpler than either of the others, so simple in fact that it was constructed with the simplest tools in half a day. It has been in use for nearly a year and, while it may not have such fine adjustments as the instruments previously described, it has proved entirely satisfactory for bacterial work.

The accompanying illustration shows its principal features. It consists of a rod, *A*, $\frac{1}{8}$ inch in diameter and 10 inches long attached at one end by a ball and socket joint, *B*, to a threaded block, *C*, which moves up and down as the screw, *D*, upon which it works is turned. To its other end is soldered a clamp, *E*, to hold the pipette. It is supported 1 inch from the clamp end by a standard, *F*, through which it passes by means of a right angle reverse bend, in such a manner as to be free to move only as the ball and socket end is raised or lowered. The standard. *F*,

about 2 inches high, is fastened to a Bausch and Lomb Type A mechanical stage, *G*, by removing the slide holder and attaching the standard in its place. The screw, *D*, operating the long end of the rod, is held in a U-shaped piece of brass, *H*, by passing

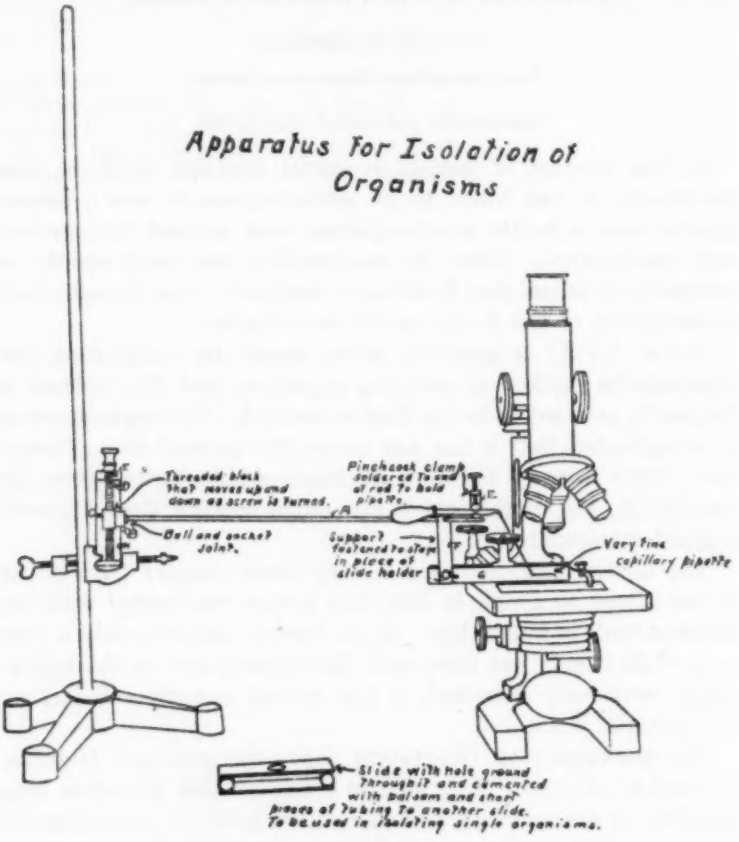
Apparatus for Isolation of Organisms
Threaded block that moves up and down as screw is turned.
Ball and socket joint.
Pinchcock clamp soldered to end of rod to hold pipette.
Support fastened to stem in place of slide holder
Very fine capillary pipette
Slide with hole ground through it and cemented with balsam and short piece of tubing to another slide. To be used in isolating single organisms.

FIG. 1

through holes in each arm of the U. The screw, *D*, has a groove cut around it where it passes through the upper arm of the U into which a small screw, *I*, in the arm fits, preventing the screw, *D*, from moving vertically but allowing it to be turned. The

U-shaped brass, *H*, is fastened to a ring stand by means of an ordinary clamp. By turning the screw, *D*, to the right or left, an up or down movement is imparted to the end of the pipette. The location of the end of the pipette may be adjusted by the rack and pinion movements of the mechanical stage. From this brief description and the illustration, the mechanism of the device may be understood.

By making the threads on screw, *D*, finer or by lengthening the rod, *A*, a finer vertical movement is obtained. The rack and pinions of the mechanical stage allow sufficiently fine adjustment for bacterial work. In order that the movements may be accurate, the threads in block, *C*, should fit the threads of screw, *D*, closely, the ball and socket joint should be carefully made and the bearing in the top of the standard, *F*, should be accurately fitted.

It is not necessary to describe the use of the apparatus since Barber and Chambers have given rather complete details as to the methods to be followed in the micropipette method of isolation.

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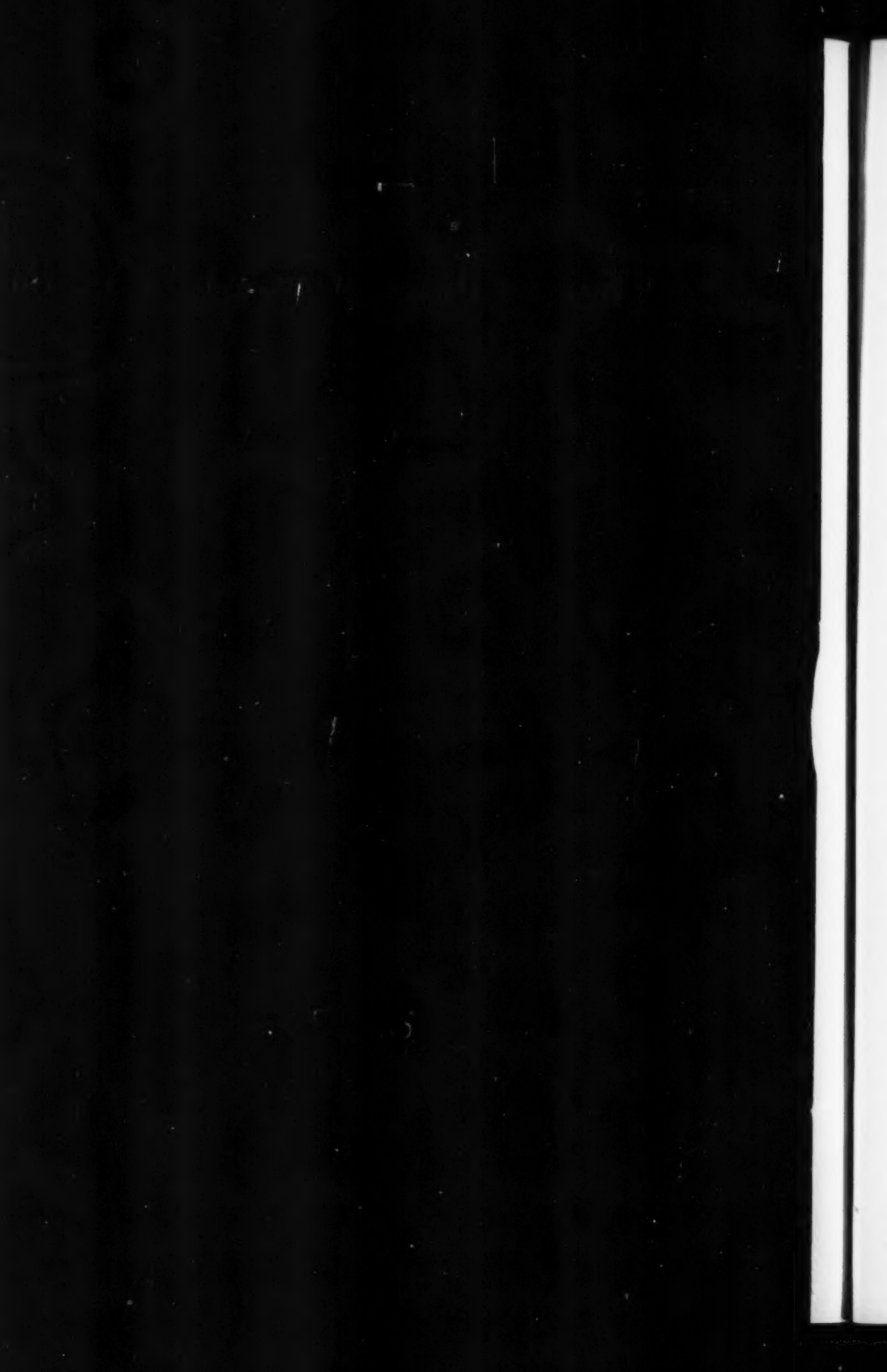

A TEST FOR INDOL BASED ON THE OXALIC ACID REACTION OF GNEZDA[1]

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Received for publication May 18, 1923

There are many methods of testing for indol, but those available for bacteriologic studies are comparatively few.

Baeyer's nitrosoindol reaction of 1870 was introduced into bacteriology by Kitasato in 1889 as a means of differentiating *Bact. coli* from *Bact. typhosum*. The method of application was that developed by Salkowski. It is still recommended in practically all text books, and is widely used in testing for indol production. The Ehrlich para-dimethylaminobenzaldehyde test for use with urine, published in 1901, was applied to bacteriology by Haenen in 1905, and by Böhme in 1906. It has become the test most frequently used for accurate work, either in its original form or with modifications such as that of Steensma.

Gnezda described a pink or purple color reaction formed by the union of oxalic acid and indol in 1899. It is not clear whether Morelli or Pittaluga first applied this reaction to bacteriologic studies. It would appear that they developed the method independently although it was some nine years after the publication of Gnezda's report. Morelli does not refer to Gnezda's work and Zipfel thought that this was simply a return to the principle of the Crisafulli pine splinter hydrochloric acid procedure. The conditions for the two tests are however quite different. Pittaluga calls the test the indol oxalic acid reaction of Gnezda. Four years before these applications to bacteriology Verschaffelt used the method to demonstrate indol from jas-

[1] Presented at the meeting of the American Association of Pathologists and Bacteriologists, Washington, May 3, 1922.

mine and orange blossoms. There are only scattered references to the method in bacteriologic literature and most of the current text books have no mention of it. Konrich is reported to have found it unsatisfactory. Zipfel, Baudet and Freund obtained results which compared favorably with other standard methods. It would appear that the method has been either overlooked or underestimated.

One of us (Holman) has used this method since 1911, and has found it very satisfactory. It was controlled by the Ehrlich-Böhme and Salkowski tests, and the results were the same in many hundreds of tests, so that it became the custom to use this test in all cases, and if it failed to show the pink color indicative of indol, to test the medium by the Ehrlich-Böhme method. There were no exceptions to the rule that both tests were negative for the same cultures.

It is generally recognized that there are so many factors in the production of indol by bacteria that the greatest precautions are necessary against drawing wrong conclusions. The results given with the Salkowski and Ehrlich tests frequently fail to agree. It would appear from the studies of Frieber and many previous workers that the Salkowski test is not reliable as a test for indol, since it gives a reaction quite similar when indol acetic acid is formed from the tryptophane molecule. The Ehrlich-Böhme test has been found to react with other compounds than indol (Zoller et al.). The amyl alcohol used for extracting the rosindol must be tested out, as many lots give a color reaction easily confused with indol (Porcher, Telle and Hüber, Baudet). Herter found that indol acetic acid reacted with para-dimethylaminobenzaldehyde, and gave a color similar to that with indol. Frieber did not find this, but rather believed that the Salkowski test is really a test for indol acetic acid. In fact, these tests have been modified and remodified in order to overcome the chances of error, and for careful work the majority of investigators rely on testing the distillates.

The more common method of distilling for indol is the complicated one by means of steam. Zoller has found direct distillation far more simple, and equally satisfactory if certain

precautions are taken. Goré has recently devised an even more direct method of taking advantage of the volatility of indol. The cotton plug of the test tube culture is dipped in the Ehrlich-Böhme solutions, pushed close to the surface of the medium, and the tube gently heated in boiling water. The volatile indol gives the characteristic color to the cotton plug. The oxalic acid test we are considering depends on the volatility of indol at 37°C. or even at room temperature.

In the unsettled state of our knowledge of these color reactions it is advisable where possible to rule out the effect of colored media such as broth, peptone water, etc., if the reagents are to be added directly to the culture media. Zipfel and others have done this in their tryptophane solutions, but we have reason to believe that such poorly buffered solutions are not adapted to the growth of all types of bacteria. The work of Frieber indicated that most of the errors in reading indol reactions are not due to other ingredients in the medium (he used Zipfel's medium modified in various ways) but to the formation by certain bacteria of indol acetic acid and possibly other compounds from tryptophane and related substances when indol is not completely liberated. Without going into detail at this time, it is enough to say that indol acetic acid is not volatile, is not found in the distillates, and will not therefore give the pink color to oxalic acid papers.

The fact that we can narrow our investigations by this simple means to the volatile compounds produced, makes this method important. The rate at which indol volatilizes is rather slow, and the concentration necessary to give the pink color to the oxalic acid paper is not known. In order to determine the dilution of indol which will give the test, 1 mgm. of pure indol was added to 1000 cc. of distilled water. Further dilutions from this stock solution were made, using as the diluting fluid Dunham's solution (Difco peptone) as suggested by Malone and Goré. A dilution containing 0.0009 mgm. per cubic centimeter could be detected after twenty-four hours in the incubator as a slight pink on the oxalic acid paper. Malone and Goré

gave 0.0025 mgm. per cubic centimeter for the ordinary Ehrlich method, 0.0005 for the Goré cotton-wool plug test, 0.0003 for Steensma's test.

The indol content in milligrams per cubic centimeter of broth as estimated by Malone and Goré after twenty-four hours, showed a continued decrease up to seven and fourteen days, undoubtedly due to the volatilizing of the indol. We have been able to test every day, by the oxalic acid papers, the indol volatilizing from cultures up to many weeks in the incubator, and have also shown that the indol is given off even after the cultures have been sterilized by disinfectants such as mercuric bichloride and choloroform. Formaldehyde stopped the reaction. Whether or not a non-volatile compound is here formed is not known. It is of no great practical importance to be able to detect indol in such short times as six hours as Rivas was able to do in his trypsinized pepton media, but it is useful to have a self recording test such as this one which gives the reading for indol when the amount reaches a certain relatively low concentration. We never had a positive test by this method when we used unseeded Dunham's tubes made up with Armour, Difco, Fairchild, Parke and Davis, Will or Witte pepton.

Zoller in a study of the influence of hydrogen ion concentration upon the volatility of indol from aqueous solution shows that the most rapid volatilization of indol takes place over a decidedly alkaline range (pH 8.0 to 10.5) which is of course of direct application to the method we are discussing. Porcher and Panisset emphasized the importance of alkalinizing the medium before distilling. As the deaminizing process in the culture continues with resulting increased alkalinity, the color change of the oxalic acid paper from the volatilizing indol becomes more rapid and distinct. The papers can readily be replaced as desired and the time of maximum volatility, but not necessarily of production, may be thus simply determined.

An interesting and as yet a not readily explainable phenomenon is the fading of the pink color on the paper after several days, which, starting at the extreme lower edge, very slowly decolorizes the whole of the exposed paper. There is some evidence that

it is due to ammonia, and tests with acid fuchsin papers give the maximum ammonia reaction at the edge of this test paper at the same time that the edge of the pink oxalic acid paper is beginning to fade. This phenomenon offers no difficulty in obtaining records, since it takes many days to decolorize the length of the paper, and even after weeks, the paper, where it is held between the glass and the cotton plug, still shows pink.

This volatility of indol can be demonstrated on filter papers, strips of white tape, or even on the absorbent cotton plugs. These are dipped in a saturated watery solution of oxalic acid and allowed to dry. The filter paper should be folded four or five times to prevent if from lying against the side of the tube and to offer a greater surface to the rising indol. The tape may curve under the cotton plug, both ends being held in place. The absorbent plugs can be lightly dipped in the saturated solution and dried in situ, care being taken not to have an excess of crystals on the cotton. It is important to remember that the reaction does not occur if the papers are wet. We have taken untreated absorbent cotton plugs from cultures of indol producing bacteria, and having shaken them in ether, have obtained from the ethereal solution a clear cut, sharp reaction for indol by the Steensma modification of the Ehrlich test. We have also taken the pink oxalic acid papers and treated them in the same way, the solution giving a sharply positive Steensma test.

It would appear that the crystals of oxalic acid must be very small, or distributed on some finely divided material. There was no color change of the oxalic acid crystals deposited on the walls of a Kjeldahl distilling tube where the volatilizing indol must have come in direct contact with the crystals, nor when the indol was allowed to pass up through packed crystals of oxalic acid in a Fresenius filter tube.

The very great advantage of being able to use this method with solid media or colored or various complex media, needs to be emphasized. We have had no trouble in demonstrating indol by a sharp pink reaction on the oxalic acid papers and oxalic acid tape above a cultre of *H. influenzae* grown on "chocolate"

(heated blood) agar. A great variety of indol producers grown on agar slants also gave sharp reactions. There is no evidence that this test for indol is less accurate than any other, and it undoubtedly eliminates a great many sources of error liable to occur with the other more commonly used tests.

The advantages of this method of demonstrating indol are many, and they may be briefly restated:

1. It depends on the volatility of indol, with no special methods of distillation. This eliminates a great many substances formed or present in the media which may give confusing color reactions where the tests are made directly in the media.

2. It leaves the tube or flask free for any other test thought necessary or desirable, so that the method is readily compared with other tests.

3. It tells the time of maximum or at least active indol concentration in the simplest way, and thereby eliminates the errors where the test is made too early or too late. The latter possibility is, however, not important, since the indol does not under ordinary conditions disappear from the media for a long time.

4. It can be used with solid media such as plain agar, heated or unheated blood agar, as well as any colored or opaque fluid media which cannot be used with the other common testing methods.

5. It eliminates the chief source of disagreement between the Baeyer-Salkowski nitroso-indol reaction and the Böhme-Ehrlich rosindol test because the compound responsible for this disagreement, indol acetic acid, is not volatile.

6. In a word, it is simple, accurate and practical.

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THE GROWTH OF YEASTS ON SYNTHETIC AGAR MEDIA

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Received for publication May 31, 1923

In the course of a study by one of the authors of yeasts found in cream and butter, a medium has been used for plating known as "whey agar." The whey was obtained from skimmed milk by coagulation with rennet. To each 100 cc. of the whey there were added 1.5 cc. of agar and 0.50 grams of pepton. The medium was filtered through absorbent cotton, tubed in 10 cc. portions, and sterilized for twenty minutes at 15 pounds pressure. One cubic centimeter of a 1 per cent tartaric acid solution was added to the Petri dish to keep down bacterial growth.

The successful use of synthetic liquid media for the growth of yeast by Fulmer, Nelson and Sherwood (1921) and Nelson Fulmer and Cessna (1921) led the authors to try to develop a satisfactory synthetic agar medium for the growth of yeast. Such a medium would offer many advantages, chief among them being the possibility of preparing media of more constant composition and properties than would be possible by the use of such variable materials as whey and pepton. These media would serve as basal in a quantitative study of the effect of various chemical environments upon different varieties of yeasts and so might lead to their differentiation and isolation.

EXPERIMENTAL

Three types of organisms were used: *Sacch. cerevisiae*, *T. sphaerica* a lactose fermenting yeast (Hammer and Cordes, 1920); and Mycoderma. Tests of the agar media were made both on plates and on slopes. The compositions of the basal media are given in table 1.

The beer wort was made by mashing, at 55°, 360 grams of ground distiller's malt with 1150 cc. of distilled water for twenty-four hours. The mixture was filtered through towelling, then through filter paper and heated for thirty minutes under 15 pounds pressure. After standing for three days to permit coagulated matter to settle out, the liquid was filtered and the agar added. The material was heated in the autoclave at 15 pounds for thirty minutes, filtered through absorbent cotton and tubed. The tubes were sterilized in live steam for thirty minutes on two successive days. The agar was washed several times in distilled water.

TABLE 1

CONSTITUENT	GRAMS PER 100 CC.				
	Medium I	Medium II	Medium III	Medium IV	Medium V
Ammonium chloride	0.188	0.188	0.188		
Dipotassium phosphate	0.100	0.100	0.100		
Calcium chloride	0.100		0.100		
Cane sugar	5.00	5.00			
Agar	1.50	1.50	1.50	1.50	1.50
Whey				Basal	
Beer wort					Basal

EFFECT OF VARIATION OF CONCENTRATION OF AMMONIUM CHLORIDE

Fulmer, Nelson and Sherwood (1921) found that optimum reproduction of yeast took place at 30° at a concentration of 0.188 gram of ammonium chloride per 100 cc. of medium. The optimum concentrations of salts in the presence of agar would not necessarily be the same as those for the liquid media.

With medium I as basal, the concentration of ammonium chloride was varied. The colonies were first counted after a period of incubation of one week at 20°C. Table 2 gives a summary of typical experiments.

The actual number of colonies is independent of the concentration of ammonium chloride and this number is practically

identical with that obtained on the whey or wort agar; the synthetic agar media give the same quantititive information as do the usual media used, whey agar and wort agar.

The colonies in the synthetic media are about $\frac{1}{2}$ to $\frac{2}{3}$ the diameter of those obtained on wort or whey agar, Mycoderma forming the largest colonies, *T. sphaerica* the smallest, with those of *Sacch. cerevisiae* intermediate. However, the colonies were of sufficient size to make counting very easy. The variation of the concentration of the ammonium chloride did not alter the diam-

TABLE 2

CONCENTRATION OF AMMONIUM CHLORIDE	NUMBER OF COLONIES		
	Sacch. cerevisiae	*T. sphaerica*	Myocoderma
0.062	41	137	46
0.093	37	151	40
0.124	33	149	32
0.155	34	149	37
0.188	34	136	35
0.217	39	151	37
0.248	33	152	45
Whey agar	38	135	40
Wort agar	36	140	36

eter of the colonies to any appreciable extent. A significant increase in the number of cells would have little effect upon the diameter of the colony. The failure to obtain significant differences in the diameters of the colonies does not in any way contradict previous statements regarding an optimum concentration of ammonium chloride for the reproduction of yeast.

A STUDY OF MEDIA I, II AND III

The three types of yeast grew well on media I and II but showed starved growth on III. Mycoderma showed the poorest growth of the three types on Medium III.

In no case, even where there was starved growth, was there a decrease in the number of colonies as compared with those produced on whey or wort agar. The yeasts were able to produce the standard number of colonies on the sugar free medium.

The energy and carbon were derived from the stored carbohydrate material or from the agar. A detailed study is being made of the stored carbohydrate material of these three types.

The colony shapes were typical of the three types on the synthetic media, these forms differing from those typical on whey agar. Table 3 illustrates the differences.

TABLE 3

TYPE	DESCRIPTION OF COLONIES
Saccharomyces cerevisiae	Circular, filamentous. The filaments are composed of long cells with branches at the internodes. On whey or wort agar the colonies are circular with sharp edges.
Mycoderma	Small, irregular, compact growth with characteristic filaments composed of long cells with rosettes of single cells at the internodes. On whey or wort agar the colonies grow as a dull film, round, showing radial formation
T. sphaerica	Small, circular, sharp edged, compact. On whey or wort agar the colonies are circular, convex, smooth, apaque, with entire edge

SUMMARY.

Synthetic agar media may be used for quantitative work with the three types of yeast studied.

Typical colony formation is shown by the three types of yeasts used.

The synthetic agar media furnish poor conditions for the growth of bacteria and are particularly suitable for the growth of yeasts.

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